The Effect of Radiotherapy on Gentamicin Ototoxicity: An Animal Model

Aren Bezdjian1,2*, Mario A. Mujica-Mota, MD, MSc1,2*, Slobodan Devic, PhD, MSc3,4, and Sam J. Daniel, MD, MSc1,2

Abstract

Objective. Patients undergoing radiotherapy (RT) often present with serious bacterial infections requiring the use of antibiotic treatment. Gentamicin is a commonly used aminoglycoside antibiotic, whose ototoxicity remains a major problem in clinical use. The objective of this study was to determine whether radiation exposure can influence gentamicin-induced ototoxicity.

Study Design. Prospective animal study.

Setting. Animal care facilities of the Montreal Children's Hospital Research Institute.

Methods. Sixteen guinea pigs received low-dose RT unilaterally for 4 weeks (total: 48 Gy). Animals then received low or high doses of gentamicin (40 mg/kg/d and 80 mg/kg/d) for 10 days. The ears were divided into 4 groups: gentamicin 40 mg, gentamicin 80 mg, gentamicin 40 mg + RT, and gentamicin 80 + RT. Auditory brainstem responses and distortion products otoacoustic emissions were assessed at baseline and before and after gentamicin treatment. Cochlear morphology using light and scanning electron microscopy were evaluated.

Results. High-dose gentamicin caused significant auditory brainstem response threshold shifts (P = .020), with greater hearing loss in the irradiated ear (difference of 23.6 ± 7.5 dB). All animals exposed to high-dose gentamicin had head tilts toward the radiated side. Cochlear morphology revealed the greatest hair cell damage in the gentamicin 80 + RT group followed by gentamicin 80.

Conclusion. Results suggest that radiation can exacerbate the ototoxicity of gentamicin at high doses.

Keywords
radiotherapy, radiation, gentamicin, aminoglycoside, guinea pig, hearing loss

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hypothesis has not been confirmed in vivo. Therefore, the objective of the present study is to determine if low-dose radiation exacerbates gentamicin-induced ototoxicity in a guinea pig animal model. Our hypothesis given the results of the previous in vitro study is that low-dose radiotherapy can predispose to gentamicin ototoxicity at low and high doses.

Materials and Methods

Animal Subjects

The study was approved and monitored by the McGill University Health Centre Animal Care Committee in accordance with the Canadian Council of Animal Care Guidelines. Sixteen 6-week-old female albino guinea pigs (450 to 500 g) purchased from Charles River Laboratories (Wilmington, Massachusetts) were kept in standard housing at 22°C ambient temperature with a 12-hour light/dark cycle. All animals had free access to food and water and were examined daily for signs of pain, weight loss, or head tilt.

Experimental Design

Bilateral baseline auditory brainstem responses (ABRs) and distortion products otoacoustic emissions (DPOAEs) were performed prior to treatment. All animals were randomly assigned an experimental ear to receive a total dose of 48 Gy of RT. Bohne et al. delivered fractionated doses of radiation in chinchillas and observed that cytocochleograms of animals subjected to doses between 40 and 50 Gy had less than 10% outer hair cell (OHC) loss and minimal nerve fiber degeneration.

Sixteen weeks after radiation treatment, the animals received gentamicin (Gentocin, Merck Animal Health, Canada) at low and high doses: 40 mg/kg/d or 80 mg/kg/d subcutaneously for 10 days. The ears of the animals were subsequently divided as followed: gentamicin 40 mg (n = 8), gentamicin 80 mg (n = 8), gentamicin 40 mg + RT (n = 8), gentamicin 80 + RT (n = 8). The high dose chosen for the study (80 mg/kg/d) has been demonstrated to cause sufficient ototoxicity yet no mortality, while low doses (40 mg/kg/d) have been shown to have no ototoxic effect. In addition, there is a current trend toward the use of a once-a-day regimen of gentamicin given its pharmacokinetic profile and its outcomes in neonatal sepsis.

Therefore, it was decided to use a single daily dose regimen in our study.

Bilateral ABRs and DPOAEs were repeated 1 week and 16 weeks after RT to assess for short- and long-term hearing deficits. Final hearing tests were performed after the 10-day gentamicin treatment to assess for hearing loss caused by gentamicin alone (control ear) and the synergistic effect of gentamicin + RT (experimental ear) at both doses.

Sample Size

A sample size of 8 animals was obtained using a power of 80%, an α of .05, and a minimum absolute difference, which represented the ABR amplitude threshold differences of 20 dB with a standard deviation of 20 dB. Therefore, 16 animals were used for the purpose of this study: 8 receiving low-dose gentamicin and 8 receiving high-dose gentamicin.

Irradiation System

A customized restrainer was constructed in concordance with Winther’s study. A protective lead shield covered the restrainer and properly guided the radiation source to the target cochlea. On the front teeth of the guinea pig were placed a horizontal wire and lateral screws to immobilize its head. The protective lead shielding covered the entire animal, leaving the collimator window to create a radiation field size of 6.5 mm × 7.2 mm at the level of the cochlea.

The animals immobilized with inhalational anesthesia and within the restrainer were positioned inside a Faxitron CP-160 Cabinet X-Radiator System (Faxitron X-Ray Corp, Wheeling, Illinois) on tray guide No. 8. A 0.5-mm Cu filter was added, and parameters were set at 160 kVp and 6.3 mA. The fraction size was 2.4 Gy per day given on the weekdays for 4 weeks, resulting in a total dose of 48 Gy, which is considered low and non-ototoxic. Using external landmarks (external ear canal and 4 mm from the midline), the beam of the x-ray tube was positioned over the experimental cochlear region. The irradiation time for each fraction was calculated using the output (1.35 Gy/min) measured (for a given setup) using the EBT model radiographic film-based reference dosimetry system, as specified in a previous study.

Assessment of Hearing

All hearing tests were conducted using inhalational anesthesia with 2% isoflurane. Once the external ear was inspected, stainless-steel needle electrodes were placed subdermally at the pinna, vertex, and contralateral pinna. Bilateral ABR testing was obtained using the SmartEP System (Intelligent Hearing Systems, Miami, Florida). Tone burst stimuli (8, 16, 20, and 25 kHz) with Blackman envelope were presented through insert earphones at 80-dB sound pressure level (SPL), decreasing in steps of 20, 10, and 5 dB to the threshold. Responses to the stimuli were amplified, filtered, and averaged over 1600 sweeps. The ABR threshold was selected at the last intensity at which 3 reproducible waves III and V could be identified. DPOAEs were obtained using the Smart DPOAE high-frequency software/hardware package (Intelligent Hearing Systems). The otoacoustic emissions were recorded for both ears between 0.8 and 22 kHz for a total of 9 frequencies. Two-tone stimuli at 55 and 65 dB SPL were emitted with a frequency ratio (F1/F2) of 1.22 and averaged 32 times. The signal-to-noise ratio (SNR) at the F2 amplitude was used to assess hearing function.

Histological Analysis

The animals were killed after the final hearing test, and cochleae were obtained. The cochleae were fixed with 10% formalin for 48 hours and decalcified with 10% EDTA in phosphate-buffered saline (0.1 M, pH 7.4) for 3 weeks at 4°C. The samples were then dehydrated in ethanol and embedded in paraffin and cut into 5-μm midmodiolar sections. Final slides were stained with hematoxylin and eosin.
and mounted for light microscopy. Images of the stria vascularis, spiral ganglion cells, and hair cells were digitally stored using AxioVision 4.7 microscopy software and a Zeiss AxioCam MR3 camera (Carl Zeiss, Jena, Germany).

For scanning electron microscopy, 3 randomly chosen cochleae from each group were fixed in 2.5% glutaraldehyde for 2 hours and then left in 0.1 M phosphate-buffered saline for 24 hours at 4°C. The cochleae were then post-fixed in osmium tetroxide for 1.5 hours and dehydrated in graded solutions from 35% to 70% ethanol. Once the organ of Corti was dissected under a surgical microscope, the samples were further dehydrated in solutions up to 100% ethanol, critical point dried, mounted, and sputter coated with gold. A field emission scanning electron microscope was used for qualitative analysis (Hitachi S4700; Hitachi, Tokyo, Japan).

Statistical Analysis

The data were analyzed using a repeated-measures analysis of variance that examined the effects of 2 within variables, time of measurement (1 week post-RT, 16 weeks post-RT, and post gentamicin) and frequency exposure (8, 16, 20, and 25 kHz), and 1 between variable, treatment (gentamicin 40, gentamicin 80, gentamicin 40 + RT, gentamicin 80 + RT). The data were analyzed using SPSS Statistics Software version 20 (IBM Corp, Armonk, New York).

Results

Behavioral Assessment of the Animals

Following the first dose of radiotherapy, the animals were examined daily for signs of vestibular toxicity, head tilts, significant weight loss, or ear infection. At the completion of radiotherapy (total dose of 48 Gy), the animals did not display head tilts, a significant reduction in physical activities or weight, or ear discharge to suggest that radiotherapy resulted in systemic disturbances. The same observations were noted in the animals treated with gentamicin 40. However, the animals receiving gentamicin 80 displayed systemic changes that became increasingly apparent throughout the 10-day treatment. All animals exposed to gentamicin 80 had head tilts on the radiated side. The animals also showed signs of reduction in physical activity and weight loss.

ABRs

The radiation dose of 48 Gy did not cause short- or long-term hearing loss as demonstrated in ABR at all frequencies tested previous to gentamicin administration (Figure 1). The statistical analysis revealed a significant main effect for the time of measurement, \( F = 47.99, P < .001 \), having greater threshold shifts in all groups after receiving gentamicin. Furthermore, an effect was observed for treatment, \( F = 3.11, P = .050 \). A significant time \( \times \) treatment interaction was observed, \( F = 5.40, P < .001 \). Post hoc comparisons of the time \( \times \) treatment interaction effect were performed using between-groups \( t \) tests. No significant differences were detected between the treatments at week 1 and 16; however, significant differences were observed after gentamicin treatment.

Post hoc comparisons of the treatment main effect, using between-groups \( t \) tests, revealed that auditory thresholds shifts were significantly higher for gentamicin 80 compared with gentamicin 40 (\( P = .036 \), showing a dose-dependent effect in the ears treated solely with gentamicin. A significantly higher threshold shift was observed in the radiated ears compared with the controls ears of animals receiving gentamicin 80 (14.54 ± 4.32 dB, \( P = .018 \); Figure 1).

The results displayed in Figure 2 compare ABR threshold shifts prior (week 16 post-RT) to gentamicin treatment and after gentamicin treatment. The radiated ears did not differ from the control ears in the animals subjected to gentamicin 40 at all frequencies tested (\( P > .05 \)). The animals subjected to high-dose gentamicin 80 had the greatest hearing threshold shift at 16 kHz when comparing the radiated to control ears (31.07 ± 9.97 dB, \( P = .002 \). The only other significant threshold shift was found at 8 kHz in the animals.

Figure 1. Auditory brainstem response threshold shifts between groups across all time points demonstrated a significant difference between gentamicin 80 + radiotherapy compared with gentamicin 80. Error bars represent standard error of the mean.

Figure 2. Auditory brainstem response threshold shifts by frequency displayed a significant difference at 8 and 16 kHz between the groups of gentamicin 80 + radiotherapy and gentamicin 80. Error bars represent standard error of the mean.
subjected to gentamicin 80 when comparing the radiated to control ears (23.50 ± 9.97 dB, \( P = .020 \)). A higher threshold shift was observed at 20 kHz and at 25 kHz; however, these results were not significant (\( P = .15 \) and \( P = .28 \), respectively).

**DPOAEs**

DPOAEs showed no significant difference between the groups (\( P > .05 \)). However, the ears treated with gentamicin 80 and subjected to radiotherapy showed a trend of higher threshold shifts across all frequencies tested, whereas the group of gentamicin 80 displayed a pattern of damage only in the higher frequencies (Figure 3). In the animals subjected to gentamicin 40, there were no significant differences noted between the experimental and control ears.

**Morphological Analysis**

The organ of Corti and stria vascularis of the ears subjected to gentamicin 40 with and without radiotherapy demonstrated vacuolization of the stria vascularis and preservation of the hair cell morphology. Contrarily, in the ears subjected to gentamicin 80, there was preservation of the strial morphology but disarrangement of the inner and outer hair cells structure. In the ears subjected to gentamicin 80 + RT, there was marked damage at both hair cell structures and stria vascularis (Figure 4).

Despite the lack of functional changes in the ears treated with gentamicin 40, qualitative evaluation of spiral ganglion cells evidenced that RT caused morphological changes in the ears subjected to gentamicin 40. Furthermore, the ears exposed solely to gentamicin 80 evidenced shrinkage of cellular bodies. The ears receiving gentamicin 80 + RT presented marked disarrangement of the cellular bodies (Figure 5).

Scanning electron microscopy revealed near complete damage in the auditory hair cells of the ears subjected gentamicin 80 + RT. In the ears subjected to gentamicin 80 alone, the stereocilia of the hair cells were damaged and absent in some areas. In the gentamicin 40 groups, the stereocilia of the hair cells had remained intact in the ears subjected to gentamicin 40 alone and revealed minimal damage in the samples receiving combined gentamicin 40 + RT (Figure 6).

**Discussion**

The functional outcomes of the present study revealed that RT acted synergistically after treatment with gentamicin 80, as demonstrated by our ABR and DPOAE results. Although not evidenced by the previously mentioned tests, RT showed detrimental effect in the gentamicin 40 group, evidenced in the histological findings. Gentamicin 40 + RT revealed hair cell and spiral ganglion cell damage, while gentamicin 40 showed complete preservation of the inner and outer hair cells (Figure 6).

To our knowledge, this is the first study assessing the predisposing effect of RT to gentamicin ototoxicity in vivo.

In pediatric patients with brain tumors who receive fractionated RT to the inner ear with a total dose greater than 30 to 50 Gy, there is a high risk of developing hearing deficits, particularly in the high-frequency hearing range.\(^3\) It is believed that this adverse effect is caused by the reactive oxygen species (ROS) produced after irradiation primarily in the mitochondria,\(^16\) which in the long term predisposes the organ of Corti to be at risk of damage. However, little is known about the effect of fractionated schemes of lower doses on the hearing structures and its long-term effect. On the other hand, aminoglycoside ototoxicity is dependent on the dosage and interindividual variability.\(^17\) Recent studies in infants and children show that hearing loss is now a rare complication of aminoglycoside therapy, occurring in 5% to 25% of patients.\(^17, 18\) Similarly to RT, its effects are widespread to hair cells and neurons through the generation of
ROS\textsuperscript{19,20} and extend to the mitochondria of the cells.\textsuperscript{21} Ultimately, this results in the destruction of the OHCs, beginning in the base and progressing to the apex, which leads to high- to low-frequency hearing deficits.\textsuperscript{22}

Consistent with our findings, other studies have established the potential predisposing effect of RT to ototoxic agents such as cisplatin. In pediatric patients, it has been established that RT predisposes to cisplatin ototoxicity.\textsuperscript{23,24} Moreover, as evidenced by Baranak et al\textsuperscript{25} and Miller et al,\textsuperscript{26} significantly greater threshold shifts were observed in animals subjected to combined therapy of RT and cisplatin in comparison with both therapies alone. Interestingly, Miller et al\textsuperscript{26} showed that fractionated RT enhanced the toxic effect of cisplatin when the latter was administered at nonototoxic doses to guinea pigs. While these clinical and animal studies stress the effects of simultaneous combined therapy for RT and other ototoxic agents, our study suggests that RT predisposed the hearing structures to damage even after a long-term recovery period.

Similarly, other authors have pointed out synergistic hearing loss of gentamicin and other ototoxic agents (Table 1). Collins\textsuperscript{36} observed that noise and gentamicin at ototoxic doses had acted synergistically, leading to greater hearing loss. Lin et al\textsuperscript{27} evidenced damage to the auditory and spiral ganglion cells in the presence of cochlear ischemia and
Table 1. Selected Studies of Gentamicin Combinations Worsening Hearing in Guinea Pigs Compared to Gentamicin Use Alone.

<table>
<thead>
<tr>
<th>Author</th>
<th>GM Dose</th>
<th>Route</th>
<th>Combined Agent</th>
<th>Dose</th>
<th>Route</th>
<th>Details</th>
<th>Hearing Loss Compared to GM Alone</th>
<th>Cochlear HC Loss Compared to GM Alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lin et al (2011)⁴²⁷</td>
<td>125 mg/kg single dose</td>
<td>sc</td>
<td>Cochlear ischemia</td>
<td>—</td>
<td>—</td>
<td>For 30 min prior to GM</td>
<td>60 dB TS, more evident in HF</td>
<td>IHC: basal turn 42.1%, second turn 42.8% OHC: basal and second turn &gt;80%</td>
</tr>
<tr>
<td>Marra de Aquino et al (2008)²⁸</td>
<td>10 mg/kg/d × 30 d im Amikacin 400 mg/kg/d × 12 d im</td>
<td>After GM</td>
<td>Reduction in OAE amplitude/intensity</td>
<td>48% more OHC loss</td>
<td>48% more OHC loss</td>
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<tr>
<td>Riggs et al (1999)²⁹</td>
<td>75 mg/kg/d × 14 d sc Metronidazole 35 mg/kg/d × 14 d sc</td>
<td>Same time as GM</td>
<td>During GM</td>
<td>10 dB CAP TS</td>
<td>15% OHC loss</td>
<td></td>
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<tr>
<td>Conlon and Smith (1998)³⁰</td>
<td>100 mg/kg/d × 30 d im Iron 2 mg/kg/d or 6 mg/kg/d × 30 d im</td>
<td>Prior, beginning, middle, and end of GM</td>
<td>CAP reduction uv at 1 kHz</td>
<td>45.9% and 73.8% more OHC loss</td>
<td>45.9% and 73.8% more OHC loss</td>
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<tr>
<td>Riggs et al (1996)³¹</td>
<td>50 mg/kg/d × 14 d sc Cisplatin 6 mg/kg/d × 14 d sc</td>
<td>Prior: no difference</td>
<td>First row OHC and some IHC</td>
<td>10% more OHC loss, 12% more IHC loss</td>
<td>10% more OHC loss, 12% more IHC loss</td>
<td></td>
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<tr>
<td>Pye and Collins (1991)³²</td>
<td>50 mg/kg single dose sc Noise 8 kHz at 116 dB SPL</td>
<td>1 h daily, 20 min after GM</td>
<td>TS</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Bhattacharyya and Dayal (1991)³³</td>
<td>200 mg/kg/d × 7 d im Noise 2 kHz at 95 dB SPL</td>
<td>2 h daily, 30 min after GM</td>
<td>—</td>
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<tr>
<td>Brummett et al (1990)³⁴</td>
<td>50 mg/kg/d × 16 d sc Vancomycin 100 mg/kg or 200 mg/kg × 16 d sc</td>
<td>During GM</td>
<td>CAP reduction uv at 1 kHz</td>
<td>45.9% and 73.8% more OHC loss</td>
<td>45.9% and 73.8% more OHC loss</td>
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<tr>
<td>Hayashida et al (1989)³⁵</td>
<td>150 mg/kg single dose im Ethacrynic acid + noise 30 mg/kg + 60 dB SPL sc or iv</td>
<td>1.5 h after GM</td>
<td>60-80 dB TS, more evident in HF</td>
<td>Almost all OHCs destroyed; IHCs were damaged</td>
<td>Almost all OHCs destroyed; IHCs were damaged</td>
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<tr>
<td>Collins (1988)³⁶</td>
<td>50 mg/kg/d × 10 d sc Noise 8 kHz at 116 dB SPL</td>
<td>1 h at day 1 after GM</td>
<td>HF loss in 33% of GPs</td>
<td>83% more GPs with complete OHC loss</td>
<td>Apical and basal OHC loss</td>
<td></td>
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<tr>
<td>Dodson et al (1982)³⁷</td>
<td>80 mg/kg/d × 5 d sc Noise 76 dB SPL × 7 d</td>
<td>During GM</td>
<td>—</td>
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Abbreviations: CAP, compound action potential; dB, decibels; GM, gentamicin; GP, guinea pig; HC, hair cell; HF, high frequency; IHC, inner hair cell; im, intramuscular; iv, intravenous; OAE, otoacoustic emission; OHC, outer hair cell; sc, subcutaneous; SPL, sound pressure level; TS, threshold shift; uv, microvolt.
gentamicin treatment. In another study, Riggs et al.\textsuperscript{11} showed that simultaneous treatment with gentamicin and cisplatin potentiated cochlear damage. Moreover, studies have demonstrated potentiation of gentamicin ototoxicity by relatively safe medications such as metronidazole and iron.\textsuperscript{29,30} A recent in vitro study suggested that RT and gentamicin had a synergistic effect when cells were exposed to both agents.\textsuperscript{9} The authors concluded that the damage was primarily caused through the intrinsic apoptotic pathway, which involves the mitochondrial metabolism. In concordance, our study proved the synergic effect of combined RT and gentamicin in vivo.

Our DPOAE findings were consistent not only with the dose-dependent effect of gentamicin but also with the predominant high-frequency damage.\textsuperscript{11} The trend observed in our DPOAE evaluation showed that ears subjected only to gentamicin 80 caused SNR shifts primarily in the high-frequency range, while the ears subjected to the combined therapy evidenced SNR shifts across both low and high frequencies. Despite the lack of statistical significance, these findings suggest that low-dose RT can potentiate the damage of high doses of gentamicin at the level of the hair cells.

The ABR testing showed a statistically significant difference at 8 and 16 kHz, with greater threshold shifts in the gentamicin 80 + RT group compared with gentamicin alone. These findings along with vestibulotoxicity and damage to the spiral ganglion cells indicate that low-dose RT can potentiate the effects of gentamicin at high doses at the neural component of the hearing loss. Moreover, the results obtained from the ears subjected to gentamicin 40 + RT revealed abnormal morphology of the spiral ganglion cells and auditory hair cells. Our histological findings are concordant with those observed in human temporal bone studies evaluating the effects of radiation or gentamicin alone where hair cells and stria vascularis are damaged.\textsuperscript{7,8} Interestingly RT’s predisposing effect also extended to lower gentamicin doses, although not evidenced by hearing tests.

Limitations of our study include the high dose per fraction scheme used for our RT model and the absence of a longer follow-up after exposure to gentamicin. This latter would have been relevant in the ears subjected to gentamicin 40 + RT where progression of functional damage could have been evidenced at a later period. It is important to note that the “low frequencies” tested (8-16 kHz) are still in the higher range of the guinea pig hearing spectrum. Thus, 20 and 25 kHz might have been over the hearing spectrum, indicating a flaw in our experimental model but explaining why we did not notice a greater threshold shift at the higher frequencies.

Conclusion

The results of the present study show that a previous exposure to low-dose RT predisposes to gentamicin ototoxicity. Hearing loss was greater in the ears subjected to high-dose gentamicin + RT compared with gentamicin alone. The ears subjected to low-dose gentamicin + RT showed only morphological evidence of cochlear damage. These findings could provide important insight about the risks of prescribing aminoglycosides to pediatric patients with previous RT to the inner ear region.

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Author Contributions

Aren Bezdjian, study design, collection, analysis, and interpretation of the data; writing and approval of the manuscript; Mario A. Mujica-Mota, study design; collection, analysis, and interpretation of the data; writing and approval of the manuscript; Slobodan Devic, study design, interpretation of data, and approval of the manuscript; Sam J. Daniel, study design, writing and approval of the manuscript.

Disclosures

Competing interests: Aren Bezdjian, supported by Fonds de Recherche en Santé du Québec (FRSQ); Slobodan Devic, supported by Fonds de Recherche en Santé du Québec (FRSQ).

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